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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	BTI-41
First Inventor or Application Identifier	Patricia L. Conklin
Title	Transgenic Plant With Increased Expression of ....
Express Mail Label No.	EL304701231

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APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231

1. [✔] * Fee Transmittal Form *(e.g., PTO/SB/17)* *(Submit an original and a duplicate for fee processing)*
2. [✔] Specification [Total Pages 24] *(preferred arrangement set forth below)*
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [✔] Drawing(s) *(35 U.S.C. 113)* [Total Sheets 5]
4. Oath or Declaration [Total Pages 2]
   a. [✔] Newly executed (original or copy)
   b. [ ] Copy from a prior application (37 C.F.R. § 1.63(d)) *(for continuation/divisional with Box 17 completed)* ***[Note Box 5 below]***
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).
5. [ ] Incorporation By Reference *(useable if Box 4b is checked)* The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. [ ] Microfiche Computer Program *(Appendix)*
7. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
   a. [ ] Computer Readable Copy
   b. [ ] Paper Copy (identical to computer copy)
   c. [ ] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. [✔] Assignment Papers (cover sheet & document(s))
9. [ ] 37 C.F.R.§3.73(b) Statement *(when there is an assignee)* [✔] Power of Attorney
10. [ ] English Translation Document *(if applicable)*
11. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
12. [ ] Preliminary Amendment
13. [✔] Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*
14. [✔] * Small Entity Statement(s) *(PTO/SB/09-12)* [ ] Statement filed in prior application, Status still proper and desired
15. [ ] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
16. [ ] Other: .....

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Applicant or Patentee: Patricia L. Conklin, Susan R. Norris and Robert L. Last
Serial or Patent No.: _____
Filed or Issued: November 16, 1999
Title: TRANSGENIC PLANT WITH INCREASED EXPRESSIO OF GDP-MANNOSE PYROPHOSPHORYLASE

Attorney's
Docket No.: BTI-41

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Boyce Thompson Institute for Plant Research, Inc.
ADDRESS OF ORGANIZATION: Tower Road, Ithaca, New York 14850

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I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled "TRANSGENIC PLANT WITH INCREASED EXPRESSIO OF GDP-MANNOSE PYROPHOSPHORYLASE", by inventor(s) Patricia L. Conklin, Susan R. Norris and Robert L. Last described in:

[X] the specification filed herewith
☐ application serial number , filed .
☐ patent number , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organi under 37 CFR 1.9(e). * NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities.

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Joyce Frank

TITLE OF PERSON SIGNING: Vice President of Operations
Boyce Thompson Institute For Plant Research, Inc.

ADDRESS OF PERSON SIGNING: Tower Road, Ithaca, New York 14850

SIGNATURE Joyce Frank DATE 11/16/95

TRANSGENIC PLANT WITH INCREASED EXPRESSION OF GDP-MANNOSE PYROPHOSPHORYLASE

REFERENCE TO PROVISIONAL APPLICATION

This application claims an invention which was disclosed in Provisional Application Number 60/126,680, filed March 29, 1999, entitled "TRANSGENIC PLANT WITH INCREASED EXPRESSION OF GDP-MANNOSE PYROPHOSPHORYLASE". The benefit under 35 USC §119(e) of the United States provisional application is hereby claimed, and the aforementioned application is hereby incorporated herein by reference.

FIELD OF THE INVENTION

The invention pertains to the field of transgenic plants. More particularly, the invention pertains to a transgenic plant with a recombinant gene for GDP -mannose pyrophosphorylase.

BACKGROUND OF THE INVENTION

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide, and the hydroxyl radical are generated by metabolic processes, chemical compounds (drugs, pesticides, or carcinogens) that are foreign to the organism, and in response to pathogens in organisms with an aerobic lifestyle. ROS are highly reactive and can oxidize biomolecules, including proteins and nucleic acids. Oxidation of fatty acids has the potential to initiate lipid peroxidation chain reactions. However, controlled oxidative responses appear to play roles in normal biological processes. An example is programmed cell death; defined in animal systems as apoptosis, and exemplified by the hypersensitive response in plants, the localized premature cell death phenomenon that characterizes

incompatible pathogen-plant interactions.

ROS are generated by a wide variety of factors in plants. Under normal conditions, ROS are generated during photosynthesis by oxygen photoreduction. High light levels can result in photooxidative damage when ROS production exceeds that of the antioxidant capacity. Such conditions occur when high light is combined with other environmental conditions such as drought, temperature extremes, or nutrient deprivation. Other factors in the plant's environment also lead to increased ROS including UV-B, air pollutants (e.g. ozone, sulfur dioxide), redox-active herbicides (e.g. paraquat), and phytotoxic metals (e.g. Zn, Cu, Cd). Plants generate ROS in oxidative bursts that occur during pathogen infection. H_2O_2 generated during oxidative bursts is thought to play an important role in initiation of the hypersensitive response, although the levels of this ROS must be tightly controlled.

As is the case with all organisms, plants have the ability to detoxify ROS. This is accomplished in part with antioxidants including thylakoid-associated α-tocopherol and carotenoids, and soluble molecules such as L-ascorbic acid (Vitamin C), glutathione (and homoglutathione), polyamines and phenolics.

Vitamin C (AsA; L-ascorbic acid) is one of the best-known plant antioxidants. AsA is present in millimolar concentrations in most plant tissues and is a crucial antioxidant and cellular reductant. As an antioxidant, AsA has the capacity to eliminate several different ROS including singlet oxygen, superoxide, and hydroxyl radicals. It also maintains the membrane-bound antioxidant α-tocopherol in the reduced state and is used as a substrate by AsA peroxidase, removing H_2O_2.

In addition to its antioxidant capacities, AsA also preserves the activity of a number of enzymes by maintaining prosthetic group metal ions in the reduced state. Although this function of AsA is well known in animal systems, it has not been widely studied in plant systems. *In vivo* evidence does indicate that AsA is necessary for the activity of the enzyme responsible for conversion of violaxanthin to zeaxanthin during conditions in which excess light energy is dissipated. Plant cell wall expansion and metabolism as well as cell division are also thought to depend at least in part on AsA. Finally, AsA can be catabolized to tartrate or oxalate in certain plant species. Given the importance of AsA in these and other roles and its abundance in all plants tested, it is

surprising that its biosynthetic pathway in plants has remained enigmatic. However, significant progress has recently been made towards the understanding of AsA biosynthesis in plants.

SUMMARY OF THE INVENTION

Vitamin C (L-ascorbic acid) acts as a potent antioxidant and cellular reductant in plants and animals. L-ascorbic acid (AsA) has long been known to have many critical physiological roles in plants yet its biosynthesis is only currently being defined. A pathway for AsA biosynthesis that features GDP-mannose and L-galactose has recently been proposed for plants. The present invention includes a transgenic plant containing a GDP-mannose pyrophosphorylase gene.

A collection of AsA-deficient mutants of *Arabidopsis thaliana* that are valuable tools for testing of a novel AsA biosynthetic pathway have been isolated. The best characterized of these mutants (*vtc1*- vitamin c) contains ~25% of wildtype AsA and is defective in AsA biosynthesis. Using a combination of biochemical, molecular, and genetic techniques, it has been conclusively demonstrated that the *VTC1* locus encodes GDP-mannose pyrophosphorylase (mannose-1-P guanyltransferase). This enzyme provides GDP-mannose, which is used for cell wall carbohydrate biosynthesis and protein glycosylation as well as for AsA biosynthesis.

In an embodiment of the invention, a genetically engineered plant includes a recombinant nucleic acid sequence encoding a protein involved in Vitamin C biosynthesis. This protein preferably encodes GDP-mannose pyrophosphorylase. The genetically engineered plant is capable of producing increased levels of Vitamin C. The plant also possesses increased resistance to environmental stresses compared to wild type plants.

In another embodiment of the invention, a genetically engineered plant includes a recombinant nucleic acid encoding GDP-mannose pyrophosphorylase. The genetically engineered plant is capable of expressing the recombinant nucleic acid. It can also

produce increased levels of Vitamin C. The genetically engineered plant has increased resistance to environmental stresses than wild type plants.

Another embodiment of the invention is a method of increasing the endogenous level of Vitamin C produced in a plant includes the overexpression of an enzyme crucial to Vitamin C biosynthesis. This enzyme is preferably GDP-mannose pyrophosphorylase. Increasing the endogenous level of Vitamin C leads to increased resistance to environmental stresses.

In another embodiment of the invention, a genetically engineered plant includes a mutant gene that encodes a form of GDP-mannose pyrophosphorylase.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the proposed pathway for L-ascorbic acid biosynthesis in higher plants. Enzymes: 1, phosphoglucose isomerase; 2, phosphomannose isomerase; 3, phosphomannomutase; 4, GDP-D-mannose pyrophosphorylase; 5, GDP-D-mannose-3,5-epimerase; 6, L-galactose dehydrogenase; 7, L-galactono-1,4-lactone dehydrogenase.

Fig. 2 shows the amount of ^{14}C-AsA expressed as a percent of ^{14}C in the total soluble fraction.

Fig. 3A shows the fine mapping of *VTC1* to a position on chromosome 2 to one side of two molecular markers.

Fig. 3B shows the sequence of a 92 kb BAC (T5I7) within the contig of Fig. 3A.

Fig 3C shows a genomic clone including ~ 1.1 kb upstream of the 5' end of the GDP-mannose pyrophosphorylase cDNA and ~0.2 kb downstream of the predicted stop codon.

Fig. 3D shows a single cytosine to thymine point mutation at position +64 relative to the first base of the presumed initiator methionine that the *vtc1-1* and *vtc1-2* mutants contain.

Fig. 4 shows the measurement of GDP-mannose pyrophosphorylase activity in extracts from both *vtc1-1* and wildtype.

Fig. 5 shows the amino acid sequences of GDP-mannose pyrophosphorylase and the mutation of a highly conserved proline to a serine at amino acid 22 in the vtc1 mutants.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Two different plant AsA biosynthetic pathways have been previously proposed; one is similar to the animal pathway while the other is quite distinct. Animals that synthesize AsA do so via the substrates D-glucose, D-glucuronic acid, L-gulonic acid, and L-gulono-1,4-lactone, which is oxidized to AsA. In the first hypothesized pathway, the carbon skeleton of the primary substrate glucose is inverted in the final product, and this inversion occurs after glucuronate formation. An analogous pathway has been proposed for plants with D-galacturonate and L-galactono-1,4-lactone as two key intermediates. However, there are strong radioactive tracer data indicating that inversion of the glucose carbon skeleton does not occur during AsA biosynthesis in higher plants which would refute the likelihood that these pathways are correct. A non-inversion pathway with the intermediates D-glucosone and L-sorbosone was also proposed. The evidence for this pathway is not very compelling, and no recent data has been published in support of it.

In vitro biochemical methods have recently generated evidence for a novel AsA biosynthetic pathway (Figure 1) that does not predict inversion of the glucose skeleton, with D-mannose and L-galactose as two key intermediates. Supporting the hypothesis that mannose is a key intermediate in the pathway, when Arabidopsis leaves are fed with [^{14}C] mannose, ~10% of the label appears in AsA by the end of a 4-h incubation. It has also been shown that [^{14}C] L-galactono-1,4-lactone could be formed when a pea embryo extract

was supplied with [^{14}C] GDP-mannose and NAD. The [^{14}C] L-galactono-1,4-lactone *in vitro*-synthesized from [^{14}C] mannose could subsequently be converted *in vitro* to [^{14}C] AsA with the addition of intact mitochondria (to supply GLDH) and cytochrome c as an electron acceptor. It has been proposed that the conversion from GDP-mannose to L-galactono-1,4-lactone proceeds occurs via L-galactose. L-galactose can be synthesized from GDP-mannose by a previously described GDP-D-mannose-3,5-epimerase activity that was detected in both pea and Arabidopsis. A previously undescribed activity (L-galactose dehydrogenase) also detected in these extracts was partially purified and shown to oxidize L-galactose to L-galactono-1,4-lactone, providing substrate for GLDH. A fascinating implication of this pathway is that it plays a key role in plant metabolism; in addition to serving as intermediates for AsA biosynthesis, intermediates in this proposed pathway are also utilized in other metabolic pathways.

The proposed AsA biosynthetic pathway (Figure 1) has branchpoints leading to both cell wall and glycoprotein biosynthesis. GDP-mannose is utilized in multiple biosynthetic processes. Both prokaryotes and eukaryotes utilize GDP-mannose in the synthesis of complex structural carbohydrates. GDP-mannose contributes to the synthesis of at least three different structural carbohydrates in plant cell walls. First, hemicellulose polymers known as mannans contain D-mannose obtained from its activated form. Secondly, GDP-mannose is the substrate for GDP-D-mannose-4,6-dehydratase, an enzyme that catalyzes the first step in GDP-L-fucose biosynthesis and encoded by the *MUR1* gene in Arabidopsis. L-fucose is present in both plant cell walls and glycoproteins. Finally, the proposed intermediate L-galactose is a minor component of the complex carbohydrates found in the noncellulose portion of the plant cell wall. In addition to a major role in structural carbohydrate biosynthesis, GDP-mannose also has a key eukaryotic role in glycosylation. In eukaryotes, most secretory and membrane proteins are glycosylated. D-mannose, the major carbohydrate component of both N- and O-linked saccharides, is transferred from GDP-mannose during the glycosylation process.

There is little known about AsA biosynthesis. In order to elucidate this process, this invention provides a method for searching for genes involved in AsA biosynthesis (VTC genes). In order to achieve this goal, mutant plants which are Vitamin C deficient are created. Then, the genes which are affected in these mutants are pinpointed. The

sequences of these genes can be determined, and compared to known sequences in a national database. Lastly, the identity of the gene(s) can be verified with the creation of recombinant plants capable of "rescuing" the mutant phenotype (AsA deficiency). By utilizing these techniques, a transgenic plant that can functionally express GDP-mannose pyrophosphorylase has been created. Also, a method of increasing Vitamin C production in a system where GDP-mannose pyrophosphorylase is a limiting factor is disclosed.

Creating Plants Mutant in AsA Biosynthesis

A plant mutant in a step leading to the biosynthesis of AsA is needed. To create this plant, a mutagenization protocol is performed. The *Arabidopsis thaliana* used in all of the experiments and the T_1 transgenics were grown in "Cornell Mix" soil (Landry, L. G., Chapple, C. C. S. & Last, R. L. (1995) *Plant Physiol.* **109,** 1159-1166).

The T_1 transgenics were grown in a light room (80-100 μmol m^{-2} sec^{-1} light provided by 400W metal halide bulbs, 20-22°C, ~25% relative humidity) under a 16 hr photoperiod. Prior to transformation by vacuum infiltration, plants were grown under a 12 hr photoperiod with other conditions as described by the said technique being known in the art, and incorporated by reference (Conklin, P.L. & Last, R.L. (1995), *Plant Physiol.* **109**, 203-212).

T_2 transgenics were germinated on sterile plant nutrient medium as described in Li, J. *et al.* (1995) *Plant Cell* 7, 447-461, and then transplanted to soil and grown under the same conditions as the T_1 transgenics. The tissues used for the tracer study and GDP-mannose pyrophosphorylase activity assay were from plants grown in a greenhouse in Exeter, U.K. as described (Conklin, P.L., *et al.* (1997) *Plant Physiol.* **115**, 1277-1285). All experiments using *vtc1-1* were performed on a line that had been backcrossed to the wildtype Col-0 progenitor four times.

The Arabidopsis *vtc1-1* mutant was isolated from EMS mutagenesized Col-0 wildtype plants by virtue of its ozone sensitivity. EMS is utilized to induce random point mutations in DNA. *vtc1-1* contains ~25% of wildtype AsA concentrations, and results strongly suggest that this deficiency is due to a defect in AsA biosynthesis. This mutant was used as a tool to identify the *VTC1* gene.

EMS (ethylene methanesulfonate) is used to induce random point mutations in DNA. Plants arising from this treatment can then be screened for a phenotype of choice (such as ozone-sensitivity or ascorbate deficiency) to isolate mutants in systems of interest. In the treatment, wildtype seeds are soaked in a solution containing EMS, rinsed several times in water, and planted in "pools" consisting of either pots or flats each containing several thousand seeds. These seeds are known as the M_1 generation (mutagenesis 1). Mutants in this generation are for the most part heterozygous for the mutation as the likelihood of the EMS mutagenizing both chromosomes (of each pair) in exactly the locale is slim. So, as most mutants of interest are "loss of function" mutants and are recessive, the M_1 seed is allowed to grow up, self-pollinate, and produce M_2 seed. If every mutation in the M_1 is recessive, one quarter of the resultant M_2 seed (from a single M_1 plant) will be homozygous for the mutation (3:1 ratio of wildtype to mutant from selfing a heterozygous plant). Each of the pools of M_2 seed (all the seed from one pot or flat) is harvested together. These different pools are then screened for the phenotype of interest.

For ozone-sensitivity, M_2 seeds are planted out at a density of ~250/6" pot and then when the plants were 2 weeks old, they are treated with 250 parts per billion ozone for 8 hrs. This treatment does not injure wildtype Arabidopsis. After 24 hrs, ozone-sensitive mutants are identified as those plants that have dead or damaged leaves. This is how the ascorbic acid deficient mutant *vtc1-1* was isolated. As ozone generates oxygen free radicals within the plant, it is not surprising that ozone-sensitive mutants (*vtc1-1*) are deficient in the antioxidant, ascorbic acid.

In order to quickly obtain additional *vtc* mutants (to get more mutant alleles of *VTC1* and *VTC2* and alleles describing new *VTC* genes) a direct screen for ascorbate deficiency is also used. A quick semi-quantitative assay for the measure of ascorbic acid is described below.

A qualitative AsA assay was developed that utilizes nitroblue tetrazolium (NBT) as a reagent for the visual detection of AsA. This new AsA assay utilizes the electron transfer dye, NBT which can be reduced by four electrons to yield the dark bluish-purple insoluble

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formazan. Purified AsA reduces NBT to the formazan, and the high AsA content in plant tissue has allowed us to take advantage of this property.

Arabidopsis leaves ~3-8 mm in length are excised and laid on a sheet of chromatography paper. Whatman 3030-6185 paper (Whatman Ltd., Kent UK) works well for this assay while generic brands do not. Each leaf is then squashed onto the chromatography paper using a curved metal weigh spatula. Ten µl of a 1 mg/ml aqueous solution of NBT (Sigma; St. Louis, MO) is then pipetted directly onto each squashed leaf. Within approximately five minutes, a bluish-purple formazan precipitate is visualized around each wildtype leaf. As the formazan tends to bleed through the chromatography paper, this precipitate can often be visualized better on the backside of the paper. The theory is that mutant plants do not contain enough ascorbic acid to convert the nitroblue tetrazolium to visible formazan.

This assay was used to directly screen ~6,000 M_2 plants and resulted in the identification of six new *vtc* mutants, one of which was *vtc1-2*. These mutant plants do not convert the nitroblue tetrazolium to visible formazan, thereby making them deficient in ascorbic acid production.

Determining Loss of Conversion from Mannose to AsA in Identified Mutants

It is well established that D-glucose is a precursor to AsA and previous results have shown that *vtc1-1* is defective in the conversion of D-glucose to AsA. As D-mannose is a biosynthetic intermediate in the newly proposed pathway (Figure 1) feeding studies were conducted to ask whether *vtc1-1* has a decreased ability to convert D-[U-^{14}C] mannose to ^{14}C-AsA. The labeling of *vtc1-1* and wildtype Col-0 leaves with D-[U-^{14}C] mannose via the transpirational stream, fractionation of the labeled extracts, and further purification of L-[^{14}C-AsA] by HPLC were done as by the said technique being known in the art, and incorporated by reference (Wheeler, G.L. *et al.* (1998) *Nature* **393**, 365-369 and Conklin, P.L. *et al.* (1997) *Plant Physiol.* **115**, 1277-1285). Briefly, excised leaves were fed with D-[U-^{14}C] mannose through the transpirational stream for 1.5 hrs and then transferred to water for 4 hr. AsA was fractionated from extracts of these labeled leaves and the amount of ^{14}C-AsA was then determined and expressed as a percent of ^{14}C in the total soluble fraction (Figure 2). A greater percentage of ^{14}C was present as L-[^{14}C] AsA in wildtype

than *vtc1-1* in every sample. Approximately 6.6% of the total ^{14}C was present as L-[^{14}C] AsA in the wildtype samples compared to ~2.6% in the *vtc1-1* samples. Therefore, the AsA-deficient mutant *vtc1-1* is defective in the conversion of D-mannose to AsA. These data strongly support the proposal that D-mannose is a substrate for AsA biosynthesis and that *vtc1-1* is defective in one of the activities responsible for conversion of mannose to AsA.

Mapping the VTC1 Locus and Sequencing the Gene

In order to determine the gene mutated in these AsA deficient plants, the *VTC1* locus was mapped onto the Arabidopsis genome with 414 *vtc1-1/vtc1-1* individuals developed from an F_2 mapping population derived from a cross with the *Ler* ecotype. Molecular markers used in this mapping included the cleaved amplified polymorphic sequence (CAPs) markers m429 and 178 and the microsatellite marker nga168.

Using a mapping population of >400 F3 families derived from a cross between *vtc1-1* and the wildtype L*er* ecotype, *VTC1* was fine-mapped to a position on chromosome 2 to one side of two molecular markers; 0.9 cM from marker m429 and 1.2 cM from marker nga168 (as shown in Figure 3A). Using microsatellite marker 178, which is >1 cM centromeric proximal to nga168, it was determined that *VTC1* is centromere distal to nga168 and m429. All seven *vtc1/vtc1* mapping lines that were recombinant between nga168 and *VTC1* were also recombinant for marker 178 (including two between m429 and nga168), indicating that the relative order of these loci is as shown. This map is inconsistent with public domain recombinant inbred results, presumably because of the limited resolution of the recombinant inbred map: m429 is reported as being centromere proximal to nga168 (http://nasc.nott.ac.uk/new_ri_map.html). Our mapping data place *VTC1* within a 2 Mb region on Chr 2 that spans m429 to just beyond marker m336, which is currently being sequenced by the Institute for Genomic Research (TIGR). The sequence of a 92 kb BAC (T5I7) within that contig (Figure 3B) was annotated by TIGR and the open reading frame T5I7.7 was identified as a putative mannose-1-phosphate guanyltransferase (www.tigr.org/docs/tigr-scripts/bac_scripts/bac_display.spl?bac_name=T5I7). An alias for this enzyme is GDP-mannose pyrophosphorylase, which catalyzes step 4 in the proposed AsA biosynthetic pathway shown in Fig. 1. In this

reaction, mannose-1-P is converted to GDP-mannose, with the consumption of GTP and the release of inorganic pyrophosphate (PPi).

Partial sequence for a GDP-mannose pyrophosphorylase cDNA, also annotated as encoding a putative mannose-1-phosphate guanyltransferase had been previously reported. The cDNA encoding the Arabidopsis GDP-mannose pyrophosphorylase (EST ID #9908, GenBank #T46645, www.ncbi.nlm.nih.gov/irx/cgi-bin/birx_doc?dbest_cu+6850) was obtained from the Arabidopsis Biological Resource DNA Stock Center (aims.cps.msu.edu/aims; Columbus, OH). This cDNA was fully sequenced on both strands. The sequence of a full-length cDNA encoding this protein defined all intron/exon borders, and this gene contains 5 exons with exon 1 and a small section of exon 2 being a 5' untranslated region. The ~40 kD protein inferred from this open reading frame has 59% amino acid identity with the mannose-1-phosphate guanyltransferase from *S. cerevisiae*. The biochemical, molecular, and genetic evidence described below supports the hypothesis that the *VTC1* vitamin C biosynthetic locus encodes a GDP-mannose pyrophosphorylase.

To test the hypothesis that *vtc1-1* and *vtc1-2* harbor mutations in the GDP-mannose pyrophosphorylase gene, the potential for mutations in the pyrophosphorylase genomic sequence derived from each of these mutant alleles was examined. The sequences of both *vtc1-1* and *vtc1-2* contain the identical single cytosine to thymine point mutation at position +64 relative to the first base of the presumed initiator methionine (Figure 3D). This predicted missense mutation would convert a highly conserved proline to a serine at amino acid 22 in the GDP-mannose pyrophosphorylase amino acid sequence (Figure 5).

The point mutation in the *vtc1* mutants does not alter the GDP-mannose pyrophosphorylase mRNA level. RNA filter hybridization analysis revealed no significant difference in the steady state level of the GMP-encoding mRNA in *vtc1-1*, *vtc1-2* and wildtype. These results are consistent with the hypothesis that the proline to serine change at amino acid position 22 affects the enzyme activity or stability, rather than transcription or mRNA stability.

The mutant alleles *vtc1-1* and *vtc1-2* were sequenced from PCR-amplification products of genomic DNAs. For each mutant allele, an ~1.4 kb BglII fragment containing

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the majority of the coding region was sequenced using the primers, 5' TGGTAAATACGCACTCAAT 3' (named 5'-GMP) and 5' AAAACAGCAAACGACCCTAACAA 3' (named 3'-GMP). To confirm the public domain sequence of BAC T5I7 that included the base mutated in the *vtc1* alleles, both strands of a portion of a Col-0 wildtype *VTC1* ClaI genomic clone (described below) were sequenced. The sequence of *VTC1*, *vtc1-1*, and *vtc1-2* that included exon 1 and intron 1 was obtained directly from genomic DNA amplified with 5'-GMP and 5' CATTCTTGTTGGAGGCTTCGG 3'. The sequence downstream of the BglI fragment for *vtc1-1* and *vtc1-2* was obtained from genomic DNA amplified with the 5' GAATAAGCATCAATCAAAACGC 3' and 5' GCTAAGACCGACTTCAATCG 3'. More than one independent PCR product was sequenced to confirm the veracity of the data.

09447359 - 111505

Determining GDP-Mannose Pyrophosphorylase Activity in the Mutant Plants

If the GDP-mannose pyrophosphorylase is mutated in this recombinant plant, then its activity should be impaired. To test this possibility, GDP-mannose pyrophosphorylase activity was assayed in the reverse direction in crude extracts that were prepared by extraction of 0.3 g of leaf tissue in 1 ml of 100 mM Tris pH 7.6, 1% PVP, 5 mM DTT, 1 mM EDTA followed by centrifugation to remove insoluble material. The reactions were performed by adding 30 ml of crude extract to 104 ml of 15.4 mM $MgCl_2$, 15.4 mM NaPPi, 13.5 mM Tris/HCl, pH 8.0, 1.1 mM EDTA and 0.1 μCi GDP-[^{14}C]-mannose (Amersham, UK), and were terminated by boiling. The reactions were clarified by centrifugation and then lyophilized. For separation of the nucleotide sugars from the sugar phosphates by thin layer chromatography, the samples were resuspended in dH_20 and a fifth of each sample was spotted onto cellulose plates (150 μm, K2 cellulose, Whatman, Clifton, NJ). The separation solvent was ethanol/1 M ammonium acetate, pH 5.0 (60:40 by volume).

To detect radioactivity, the thin layer chromatography plates were scanned with a Berthold Linear Analyzer (Berthold LB2832, Hemstead, U.K.). The identification of nucleotide sugars and sugar phosphates were determined first by comparison to a co-migrating GDP-[^{14}C]-mannose standard and second by staining plates with an ammonium

molybdate stain by the said technique being known in the art, and incorporated by reference (Dawson, R.M.C. *et al.* (1986) in *Data for Biochemical Research, Third Edition*, Oxford Univ. Press, London, pp. 485-486). The nucleotide sugars and sugar phosphates were scrapped off the cellulose plates and eluted from the cellulose in dH_20. The free sugars were released by hydrolysis and analyzed as described in Wheeler, G.L. *et al.* (1998) *Nature* **393**, 365-369. Protein concentrations were determined by the Bradford assay with γ-globulin as a control.

The Arabidopsis leaf extracts contained a potentially interfering phosphodiesterase activity that produced mannose-1-P and GMP from GDP-mannose. However this phosphodiesterase activity was completely inhibited by the high PP_i concentration used in the pyrophosphorylase assay. This inhibition of phosphodiesterase by PP_i was confirmed by experiments with bovine intestinal mucosa phosphodiesterase 1 (Sigma, St. Louis, MO) under the same conditions as the pyrophosphorylase assay.

If *VTC1* encodes GDP-mannose pyrophosphorylase, the AsA-deficient mutant *vtc1-1* would be predicted to have reduced enzyme activity compared with wildtype plants. As the activity of this enzyme is fully reversible *in vitro*, pyrophosphorylase activity can be assayed by monitoring the production of mannose-1-P from GDP-mannose and PPi by the said technique being known in the art, and incorporated by reference (Szumilo, T. *et al.* (1993) *J. Biol. Chem.* 268, 17943-17950). This assay was used to measure GDP-mannose pyrophosphorylase activity in extracts from both *vtc1-1* and wildtype. The time-dependent production of mannose-1-P from GDP-mannose and PPi is lower in extracts from *vtc1-1* than wildtype. After a 90 minute incubation, ~35% less mannose-1-P is formed in *vtc1-1* compared to wildtype (Figure 4).

Rescue of the Mutant Phenotype by Creating A Recombinant Plant

By introducing the wild type version of the GDP-mannose pyrophosphorylase gene, the mutant phenotype should be rescued. In effect, the recombinant plant created via transformation will be able to functionally express recombinant GDP-mannose pyrophosphorylase and restore function.

A 5.4 kb ClaI fragment containing the *VTC1* locus was subcloned from BAC T5I7. A 3.4 kb fragment from this subclone was then ligated into the binary vector pGPTV-BAR/HindIII by the said technique being known in the art, and incorporated by reference (Becker, D. *et al.* (1992) *Plant Mol. Biol.* 20, 1195-1197). This construct (g*VTC1*-pGPTV) was transformed into *Agrobacterium tumefaciens* pMP90 strain GV3101 and introduced into *vtc1-1* plants by vacuum infiltration.

The vacuum filtration method for transformation is discussed below. The seeds are planted on top of window screen covered soils. After the plants have bolted, clip off the primary bolt to encourage growth of secondary bolts. Perform infiltration around four days after clipping. Start a 20 ml overnight culture of Agrobacterium carrying the gVTC1-GPTV construct including the appropriate antibiotics (kan, rif, and gm) two days prior to transformation. The day before the transformation, use this overnight culture to inoculate a large (~500 ml) culture. After 24 hrs of growth, harvest cells by centrifugation and wash once with growth media without antibiotics. Resuspend bacteria at 0.8 OD units in infiltration media. One liter of infiltration media consists of 0.5X MS salts, 1X B5 vitamins, 5% sucrose, 0.044 uM benzylamino purine, 0.03% Silwet L-77, and 0.5 g MES (pH to 5.7 with KOH). Pour some of diluted bacteria into a rubbermaid dish that fits inside the vacuum oven (be sure to turn oven temperature off prior to use). Invert pot with plants to be infiltrated into culture and place in vacuum oven. Infiltrate 5-10 min at 15 in^3 Hg. The vacuum is not necessary as just dipping the plants into the culture for ~5 min also gives similar transformation frequency. For the p*VTC1*-pGPTV infiltrations, both vacuum infiltration and dipping alone produced similar results. Release the vacuum and remove the pot. Cover with plastic wrap and return to the light room. Remove the cover the next day. A newer streamlined procedure being known in the art, and incorporated by reference (S.J. Clough and A.F. Bent, 1998. *Plant J.* **16**:735-743) can alternatively be used for transformation.

Glufosinate-ammonium resistant T_1 transgenic individuals were selected by sowing seeds and spraying the soil surface with 500 ml per m^2 of 0.25 mg ml^{-1} commercially formulated glufosinate-ammonium (Finale; AgrEvo, Montvale NJ). Twelve days after sowing, resistant T_1 seedlings were transplanted to nontreated soil and allowed to self-pollinate.

T_2 progeny were scored for glufosinate-ammonium resistance by painting individual leaves with the herbicide (150 μg ml^{-1} glufosinate-ammonium, 250 nl ml^{-1} Silwet). These plants were also scored for wildtype or mutant (deficient) levels of AsA by a nitroblue tetrazolium-based method in which single leaves are squashed onto chromatography paper and treated with 1mg/ml of nitroblue tetrazolium. The AsA in wildtype leaves is sufficient to reduce the nitroblue tetrazolium to the visible precipitate formazan, while no readily visible formazan is produced upon treatment of *vtc1-1* leaves (Conklin *et al.*, in preparation). AsA levels were then confirmed by a previously described spectrophotometric-based assay (Conklin, P.L. *et al.* (1996) *Proc. Natl. Acad. Sci.* USA **93**, 9970-9974).

If the VTC1 locus encodes GDP-mannose pyrophosphorylase, a wildtype copy of this locus introduced as a transgene will complement the *vtc1-1* allele and restore normal levels of AsA. To test this hypothesis, a genomic clone including ~ 1.1 kb upstream of the 5' end of the GDP-mannose pyrophosphorylase cDNA and ~0.2 kb downstream of the predicted stop codon (Figure 3c) was subcloned from BAC T5I7 and transformed into *vtc1-1* plants by the *Agrobacterium tumefaciens* vacuum infiltration method. T_1 transgenic plants were selected by glufosinate-ammonium resistance conferred by the BAR gene.

Table 1. Cosegregation of elevated AsA levels and the selectable marker in *vtc1-1* lines transformed with genomic copy(s) of the *VTC1* locus.

Line	AsA+[1] (# Basta^{R2}/ total)	AsA- (# Basta^{R}/total)
1	79 (10/10)	28 (1/11)
2	70 (10/10)	34 (0/12)
3	75 (11/11)	29 (0/10)

[1]AsA levels were scored using a nitroblue tetrazolium-based visual method in three independent lines of segregating T_2 generation plants obtained from self-pollination of glufosinate-ammonium resistant T_1 individuals. Two week old plants were scored as AsA positive (+) if a single leaf treated with nitroblue tetrazolium (1 mg/ml) produced a visually similar amount of formazan (from reduction by AsA) as wt, while plants were scored as AsA negative (-) if the formazan precipitate was virtually absent. For each T_2 line, 10-12 individuals from both AsA (+) and AsA (-) classes were then tested for resistance to the selectable marker.

[2]Basta^{r}, glufosinate-ammonium resistant

Thirteen glufosinate-ammonium resistant T_1 transgenics that were confirmed to contain the BAR gene by PCR-amplication all contained wildtype levels of AsA. These results were consistent with the hypothesis that the transgene complemented *vtc1-1*. The T_1 lines were allowed to self-pollinate and three selected T_2 lines from independent T_1 lines were tested for co-segregation of wildtype levels of AsA (scored using a qualitative AsA assay) and glufosinate-ammonium resistance. Introduction of the *VTC1* locus into the AsA-deficient *vtc1-1* mutant confers increased levels of AsA that co-segregate with the selectable marker (Table 1). Finally, ten individuals that scored as wildtype for AsA from each T_2 line were pooled, extracts were prepared, and total AsA was measured using a quantitative spectrophotometric assay. These pooled extracts contained between 2.4 and 3.8 μmoles AsA/g FWT of AsA which is similar to the 3.1 μmoles AsA/g FWT seen in

wildtype, and greater than the 0.9 μmoles AsA/g FWT in the mutant. Together, these results confirm that the *VTC1* locus encodes a GDP-mannose pyrophosphorylase structural gene.

Applications of the Technology

GDP-mannose pyrophosphorylase is an enzyme in the recently proposed plant AsA biosynthetic pathway (Figure 1). This invention provides conclusive evidence that GDP-mannose pyrophosphorylase is encoded by the *VTC1* locus in Arabidopsis, and that the enzyme is a critical component of the AsA biosynthetic pathway. First, the AsA-deficient *vtc1-1* mutant is defective in the conversion of mannose to AsA. Second, the activity of GDP-mannose pyrophosphorylase is lower in extracts from *vtc1-1* than wildtype. Third, the *VTC1* locus genetically maps to a region of genomic DNA encoding a GDP-mannose pyrophosphorylase homologue and the *vtc1-1* and *vtc1-2* mutants each harbor the identical point mutation that alters a highly conserved proline residue in this gene. Finally, a transgene encoding the wildtype pyrophosphorylase genetically complements the *vtc1-1* mutation, increasing the AsA in the transgenic *vtc1-1* lines to levels similar to wildtype. These results demonstrate that the AsA biosynthetic pathway proposed based on *in vitro* biochemical data operates *in vivo*.

The AsA-deficient Arabidopsis mutants isolated are unique and ideal tools for the testing of this pathway. The *VTC1* locus described by one of these AsA-deficient mutants has been cloned here. As the first genetically identified plant AsA biosynthetic gene, *VTC1* has already proved the efficacy of this approach. Armed both with the knowledge of this proposed pathway and AsA-deficient mutant lines, other biosynthetic genes can be readily isolated and characterized.

There is existing evidence to suggest that increasing the AsA content of plants will be advantageous for protection against environmental sources of ROS. The AsA-deficient mutant *vtc1* is highly sensitive to O_3, a potent generator of ROS in the plant. This sensitivity can be abolished by treatment of the mutant with exogenous AsA prior to the start of the fumigation (Conklin, P. L., *et al.* 1996. *Proc Natl Acad Sci USA* **93:** 9970-9974). This pretreatment increases the concentration of AsA in *vtc1* 5-20X. Similarly, the AsA levels can also be raised in wildtype Arabidopsis at least 5-6X. Increasing the AsA

level in the plant also abolishes the O_3-sensitivity of *soz* (sensitive to ozone) mutants that synthesize wildtype levels of AsA. Therefore, increased AsA levels have the capacity to cross-protect lines with sensitivities not correlated to an AsA-deficiency. In the literature there are several other examples of a correlation between artificially increased AsA and decreased sensitivity to O_3 including one published almost four decades ago. In this experiment, a sensitive tobacco variety (Bel-W3) that is not AsA-deficient was pretreated with AsA prior to O_3 fumigation. This pretreatment increased the AsA level in this sensitive variety and decreased its O_3 sensitivity (Menser, H. A., 1964. *Plant Physiol* **39:** 564-567). In a more recent study, pretreatment of barley leaves with AsA protected both plasma membrane permeability and the light regulation of Rubisco from O_3 damage (Mächler, F., *et al.*, 1995. *J. Plant Physiol* **147:** 469-473.). Studies with the air pollutant sulfur dioxide have also shown a positive relationship between application of exogenous AsA and increased resistance to this source of ROS (Pandya, N., and S. J. Bedi, 1990. *Adv Plant Sci* **3:** 171-177). Since increased AsA clearly protects sensitive varieties from the ROS produced from air pollutants such as O_3 and sulfur dioxide, the present invention will be crucial in the development of tools for manipulating increased AsA levels.

The identification of genes involved in plant AsA biosynthesis provides us with tools to increase the endogenous AsA levels in transgenic plants. Overexpression of Arabidopsis GDP-mannose pyrophosphorylase in plants where this enzyme is a limiting factor results in increased synthesis of GDP-mannose, a key intermediate in AsA biosynthesis. *VTC1/vtc1* heterozygotes exhibit a gene dosage effect, having intermediate levels of AsA. This shows that the GDP-mannose pyrophosphorylase activity is limiting for AsA biosynthesis. Overexpression of *VTC1* in plants results in increased AsA levels. In addition to having increased nutritive value, such transgenic plants will have increased resistance to a number of environmental stresses.

The teachings of the present invention can be used as tools for use in improving the nutritional quality and environmental stress resistance of agronomically important plants as well as serving as plant-specific herbicide targets. Increased environmental stress tolerance alone could result in economic benefits from increased yield as many common adverse conditions including drought, chilling, high light, heavy metals, UV-B, and air pollutants produce damaging ROS. Basic plant metabolic pathways are normally highly

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conserved among different plant species. If AsA levels can be increased by overexpression of AsA biosynthetic genes in Arabidopsis, the technology is readily transferable to agronomically important crop plants by known methods in the art.

Accordingly, it is to be understood that the embodiments of the invention herein described are merely illustrative of the application of the principles of the invention. Reference herein to details of the illustrated embodiments are not intended to limit the scope of the claims, which themselves recite those features regarded as essential to the invention.

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What is claimed is:

1. A genetically engineered plant, or portion thereof, comprising a recombinant nucleic acid sequence that encodes a protein involved in Vitamin C biosynthesis.

2. The genetically engineered plant of claim 1 wherein said plant, or portion thereof, is a dicot.

3. The genetically engineered plant of claim 1 wherein said genetically engineered plant is *Arabidopsis thaliana*.

4. The genetically engineered plant, or portion thereof, of claim 1 wherein said nucleic acid comprises a polynucleotide that encodes GDP-mannose pyrophosphorylase.

5. The genetically engineered plant of claim 1 wherein said genetically engineered plant, or portion thereof, is capable of overexpressing said recombinant nucleic acid.

6. The genetically engineered plant of claim 1 wherein said genetically engineered plant, or portion thereof, is capable of producing increased levels of Vitamin C.

7. The genetically engineered plant of claim 1 wherein said genetically engineered plant, or portion thereof, has increased resistance to environmental stress compared to a plant of the same species without said recombinant nucleic acid wherein said environmental stress is selected from the group consisting of:

a) drought;

b) cold;

c) UV radiation;

d) air pollution;

e) salts;

f) heavy metals; and

g) reactive oxygen species.

8. The genetically engineered plant of claim 1 wherein said genetically engineered plant, or portion thereof, is edible.

9. A genetically engineered plant, or portion thereof, comprising a recombinant nucleic acid that encodes GDP-mannose pyrophosphorylase.

10. The genetically engineered plant of claim 9 wherein said genetically engineered plant, or portion thereof, is a dicot.

11. The genetically engineered plant of claim 9 wherein said genetically engineered plant is *Arabidopsis thaliana*.

12. The genetically engineered plant of claim 9 wherein said genetically engineered plant, or portion thereof, is capable of overexpressing said recombinant nucleic acid.

13. The genetically engineered plant of claim 9 wherein said genetically engineered plant, or portion thereof, is capable of producing increased levels of Vitamin C.

14. The genetically engineered plant of claim 9 wherein said genetically engineered plant, or portion thereof, has increased resistance to environmental stress compared to a plant of the same species without said recombinant nucleic acid wherein said environmental stress is selected from the group consisting of:

a) drought;

b) cold;

c) UV radiation;

d) air pollution;

e) salts;

f) heavy metals; and

g) reactive oxygen species.

15. The genetically engineered plant of claim 9 wherein said genetically engineered plant, or portion thereof, is edible.

16. A method of increasing the endogenous level of Vitamin C produced in a plant, or portion thereof, comprising overexpression of an enzyme crucial to Vitamin C biosynthesis.

17. The method of claim 16 wherein said enzyme is GDP-mannose pyrophosphorylase.

18. The method of claim 16 wherein said plant, or portion thereof, is a dicot.

19. The method of claim 16 wherein said plant is *Arabidopsis thaliana*.

20. The method of claim 16 wherein said plant, or portion thereof, comprises increased antioxidation capacity.

21. The method of claim 16 wherein said plant, or portion thereof, has increased resistance to environmental stress compared to a plant of the same species without said recombinant nucleic acid wherein said environmental stress is selected from the group consisting of:

a) drought;

b) cold;

c) UV radiation;

d) air pollution

e) salts;

f) heavy metals; and

g) reactive oxygen species.

22. The method of claim 16 wherein said method produces a plant, or portion thereof, which is edible.

23. A genetically engineered plant comprising a mutant gene that encodes a form of GDP-mannose pyrophosphorylase.

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ABSTRACT

The present invention includes a transgenic plant containing a GDP-mannose pyrophosphorylase gene. A pathway for AsA biosynthesis that features GDP-mannose and L-galactose has recently been proposed for plants. A collection of AsA-deficient mutants of *Arabidopsis thaliana* that are valuable tools for testing of a novel AsA biosynthetic pathway have been isolated. The best characterized of these mutants (*vtc1*-vitamin c) contains ~25% of wildtype AsA and is defective in AsA biosynthesis. Using a combination of biochemical, molecular, and genetic techniques, it has been conclusively demonstrated that the *VTC1* locus encodes GDP-mannose pyrophosphorylase (mannose-1-P guanyltransferase). This enzyme provides GDP-mannose, which is used for cell wall carbohydrate biosynthesis and protein glycosylation as well as for AsA biosynthesis.

Docket No. 1169

669777 "BTETHh60

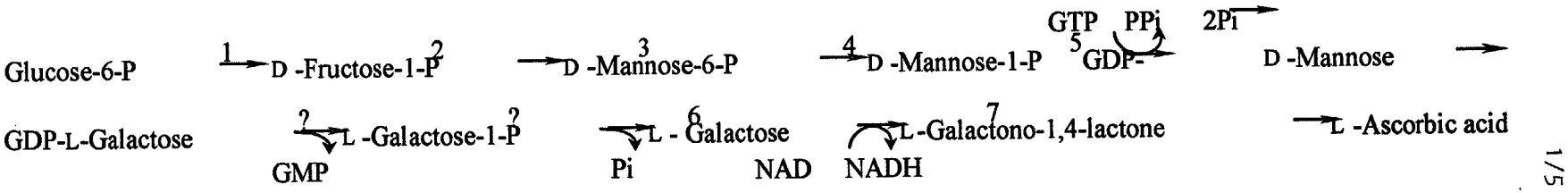


Figure 1

Figure 2

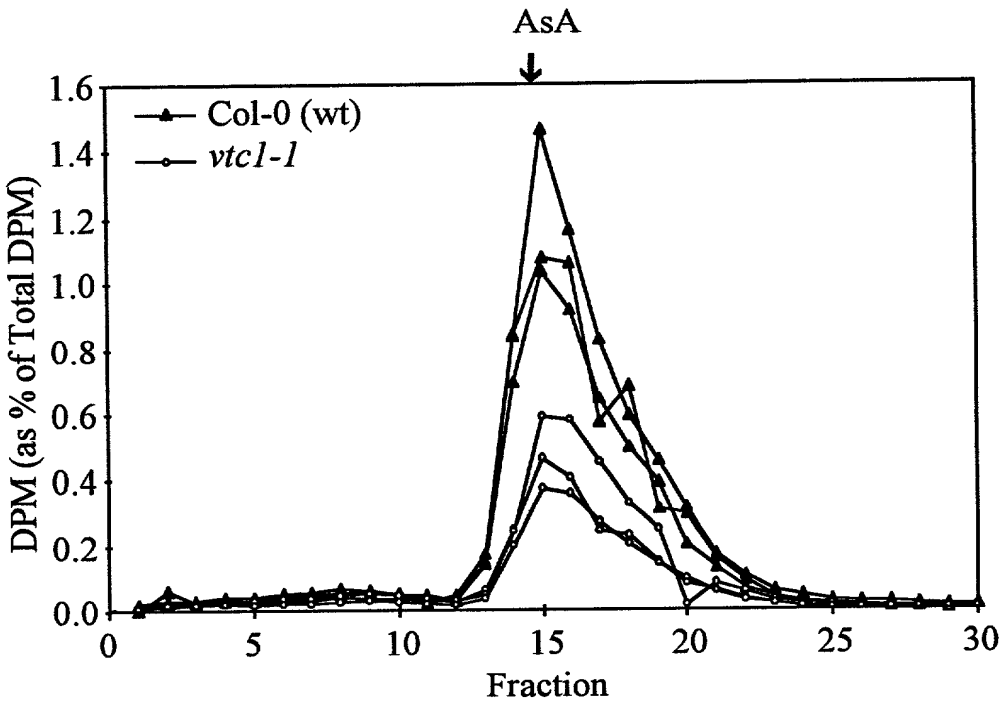

AsA
Col-0 (wt)
vtc1-1
DPM (as % of Total DPM)
Fraction
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
5
10
15
20
25
30

Figure 3

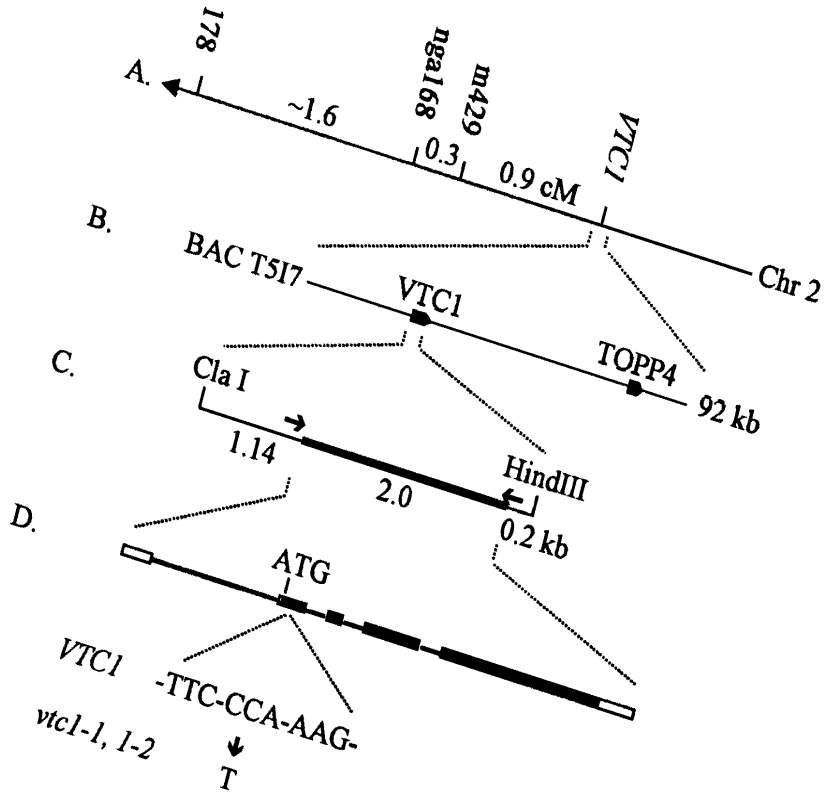
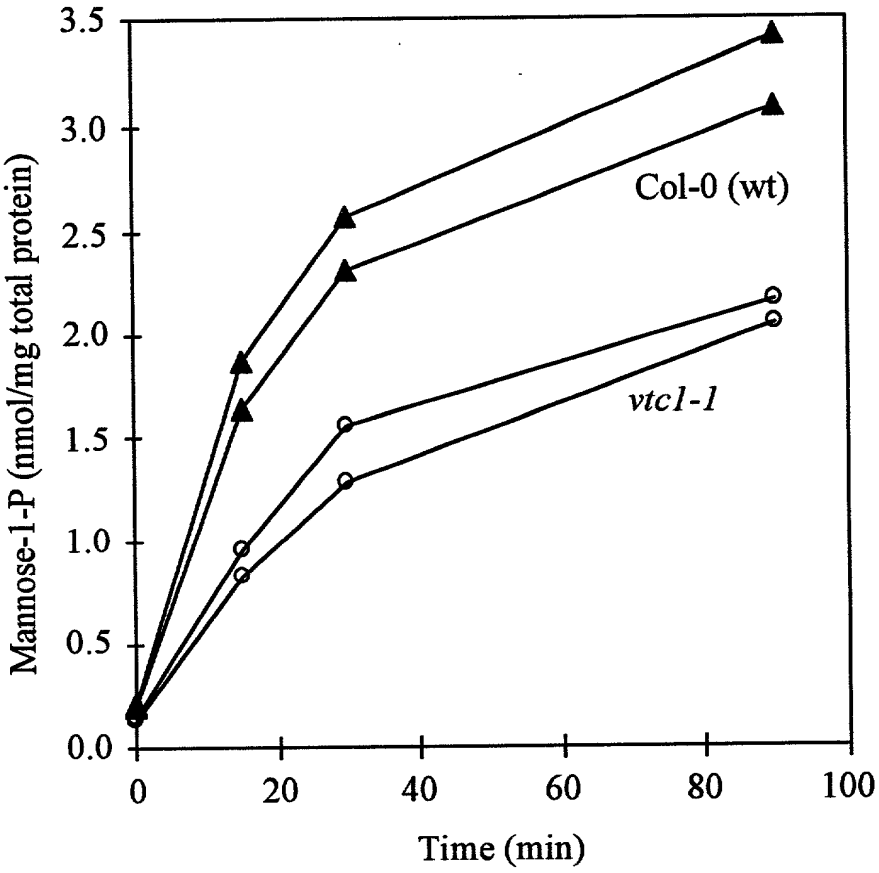

A.
178
~1.6
ngai68
0.3
m429
0.9 cM
VTC1
Chr 2
B.
BAC T5I7
VTC1
TOPP4
92 kb
C.
Cla I
1.14
2.0
HindIII
0.2 kb
D.
ATG
VTC1
-TTC-CCA-AAG-
vtc1-1, 1-2
T

Figure 4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3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Time (min)
Col-0 (wt)
vtc1-1

Arabidopsis +3
Porcine
S. cerevisiae
Synechocystis
Acetobacter
Aquifex
E. coli
Vibrio
Salmonella
Yersinia
Xanthomonas
Mycobacterium

Figure 5

Docket No. BTI-41

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

- My residence, post office address and citizenship are as stated below next to my name.
- I believe I am an original, first and joint Inventor of the subject matter which is claimed and for which a patent is sought on the Invention entitled "TRANSGENIC PLANT WITH INCREASED EXPRESSION OF GDP-MANNOSE PYROPHOSPHORYLASE", the specification of which

  [X] is attached hereto

  [ ] was filed on as
  - [ ] United States Application Number
  - [ ] PCT International Application Number

  [ ] was amended on

- I hereby state that I have reviewed and understand the contents of the above-identified application, including the claim and the drawing.
- I acknowledge the duty to disclose information which is material to patentability, as defined in 37 CFR §1.56. If this application claims priority from a prior application, I acknowledge the duty to disclose information which is material to patentability, as defined 37 CFR §1.56, which became available between the filing date of the prior application and the date of this application.
- As to the subject matter of this application:
  a. I do not know and do not believe that the same was ever known or used in the United States of America before my invention thereof;
  b. I do not know and do not believe that the same was ever patented or described in any printed publication in any country before my invention thereof, or more than one year prior to the date of this application;
  c. I do not know and do not believe that the same was in public use or on sale in the United States of America more than one year prior to the date of this application; and
  d. Said subject matter has not been patented or made the subject of an inventor's certificate issued in any country foreign to the United States of America on an application filed by me or my legal representative or assigns more than twelve months prior to the date of this application.

[X] I hereby claim the benefit under 35 USC §119(e) of any United States provisional application listed below.

Application Number	Filing Date (MM/DD/YYYY)
60/126,680	3/29/99

Please associate this application with USPTO Customer Number 020808

- I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or

imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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